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Nucleotide Sequence Variation in the Hypervariable Region of the Hepatitis C Virus in the Sera of Chronic Hepatitis C Patients Undergoing Controlled Interferon-α Therapy

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Ten patients with hepatitis C virus (HCV) infection (experimental group) were treated with interferon-α (IF-α). Dosage was six million units per day for one week and then three times a week for another six months. Seven HCV-infected patients (control group) did not receive IF-α therapy. The hypervariable region (HVR) of HCV in the sera of patients was amplified by reverse trancription-polymerase chain reaction (RT-PCR), and the variation of amino acid sequence in this region was determined. Serum alanine aminotransferase (ALT) activities in five patients treated for six months with IF-α fell to the normal range, when HCV was not detected in the sera of three patients. The nucleotide sequence variation in HVR of HCV in the sera of five patients who responded well to the IF-α therapy was relatively less than that in another five patients who did not respond to IF-α therapy and those in the control patients. These results indicate that the effectiveness of IF-α therapy was related to the sequence variation of HVR of HCV. This may have resulted from the selection pressure by humoral antibodies directed to HVR of HCV. It is concluded that the higher rate of sequence variation in HVR of HCV was compatible with a lower degree of effectiveness of IF-α therapy.

KEY WORDS: HCV, HVR, mutation, interferon-α Therapy

INTRODUCTION

Identification of the hepatitis C virus (HCV) was initially made by cloning cDNA from the HCV RNA in the plasma of a chimpanzee with chronic non-A, non-B hepatitis. HCV is now recognized as a major causative agent of non-A, non-B hepatitis [Choo et al., 1989; Kuo et al., 1989]. Acute post-transfusion hepatitis due to HCV results in chronic hepatitis in more than 50% of the cases during a one-year period. Twenty to 50% of patients progressed to liver cirrhosis [Tremolada et al. 1992; Viladomiu et al., 1992; Yousuf et al., 1992]. HCV is also related to the pathogenesis of hepatocellular carcinoma [Bruix et al., 1989; Colombo et al., 1989; Kew et al., 1990; Kiyosawa et al., 1990; Tanaka et al., 1994].

Interferon-α (IF-α) has been used to treat HCV-induced hepatitis but has been effective in only about half of the patients [Gomez et al., 1990; Hagiwara et al., 1992]. Serum alanine aminotransferase (ALT) levels returned to normal levels in 30–60% of interferon-treated patients, while the remainder did not respond to the therapy [Davis, 1990; Marcellin et al., 1991; Hagiwara et al., 1993]. Differing responses to IF-α treatment in HCV infection may be influenced by HCV subtype [Takada et al., 1993; Tsubota et al., 1994] as well as HCV RNA titer at the beginning of IF-α therapy [Naito et al., 1994].

HCV, like other RNA viruses, exhibits considerable genomic diversity [Enomoto et al., 1990; Ogata et al., 1991; Chan et al., 1992; Okamoto et al., 1993; Simonds et al., 1993; Dusheiko et al., 1994] and is capable of spontaneous mutation [Abe et al., 1992]. Mutations were more frequent in the hypervariable region (HVR) of the HCV gene corresponding to 27 amino acid residues located at the amino terminus of envelope gene 2 and nonstructural gene 1 (E2/NS1). It has been suggested that mutation at HVR of HCV is an adaptive process to escape from the host immune system for continuous propagation [Kato et al., 1994; Honda et al., 1994]. We investigated the influence of IF-α therapy on a nucleotide sequence variation in HVR of HCV in chronic HCV

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TABLE I. Nucleotide Sequences of Primers

Region	Primer direction	Sequence (5′ to 3′)	Nucleotide no.[a]
5′-UTR	outer sense	CCACCATAGATCACTCCCCTGT	57→78
	outer antisense	TTGAGGTTTAGGATTCGTGCTCAT	405→384
	inner sense	CTGTGAGGAACTACTGTCTTCA	75→96
	inner antisense	ACTCGCAAGCACCCTATCAGGC	352→331
Core[b]	outer sense (I–IV)	CGCGCGACTAGGAAGACTTC	480→499
	(V)	CGCGCGACGCGTAAAACTTC	480→499
	outer antisense	ATGTACCCCATGAGGTCGGC	781→762
	inner sense (I–IV)	AGGAAGACTTCCGAGCGGTC	489→508
	(V)	CGTAAAACTTCTGAACGGTC	489→508
	inner antisense (I)	GGATAGGCTGACGTCTACCT	537→518
	(II)	GAGCCATCCTGCCCACCCCA	632→613
	(III)	CCAAGAGGGACGGGAACCTC	662→643
	(IV)	ACCTCGTTTCCGTACAGAG	611→592
	(V)	GCTGAGCCCAGGACCGGTCT	576→557
HVR	outer sense	CACCGCATGGCTTGGGATATGATG	1331→1354
	outer antisense	CAACAGGGCTTGGGGTGAAGCA	1927→1906
	inner sense	ATGGCTTGGGATATGATGATGAAC	1336→1359
	inner antisense	AAGCAGTCGACTGGACCACACAC	1910→1888

[a]The nucleotide no. indicates the nucleotide sequence number.
[b]Core region primers for HCV typing were synthesized according to the method of Okamoto et al. [1993].

patients in order to determine the relationship between sequence variation of HVR of HCV and the therapeutic effect of IF-α.

MATERIALS AND METHODS

Specimens

Serum samples were collected from 17 HCV-infected patients who had been admitted to Severance Hospital. Sera were screened for anti-HCV antibody by enzyme immunoassay (EIA) and were confirmed with recombinant immunoblot assay (RIBA, Lucky Biotech, Korea). Histological findings indicted that all had chronic active hepatitis (CAH).

Ten HCV-positive patients were treated with IF-α (Intermax α, Lucky Biotech) with 6 million units daily for one week and then three times a week for 6 months. Seven HCV control patients did not receive IF-α treatment. All samples were prepared aseptically and were stored at −70°C until use. HCV RNA was extracted from serum with the modified method described by van der Poel et al. [1991]. After ethanol precipitation, each RNA pellet was dissolved in 10 μl of DEPC (diethylpyrocarbonate)-treated distilled water.

Preparation of HCV cDNA and Amplification

Polymerase chain reaction primers for the amplification of various regions of HCV were synthesized using a DNA synthesizer (Applied Biosystems, 381A, Foster City, CA) and purified by the method of Sambrook et al. [1989]. The primer sequences for 5′-UTR (untranslated region), HCV typing, and HVR are listed in Table I.

For the synthesis of cDNA of HCV, an aliquot of RNA (10μl) isolated from the sera of patients was mixed with 15 pmole (0.7 μl) of outer antisense primer, 2 μl of reaction buffer (250 mM Tris-HCl pH 8.3, 250 mM potassium chloride, 50 mM magnesium chloride, 50 mM dithiothreitol, and 2.5 mM spermidine) and 7.3 μl DEPC-treated water. After the contents were heat-treated for

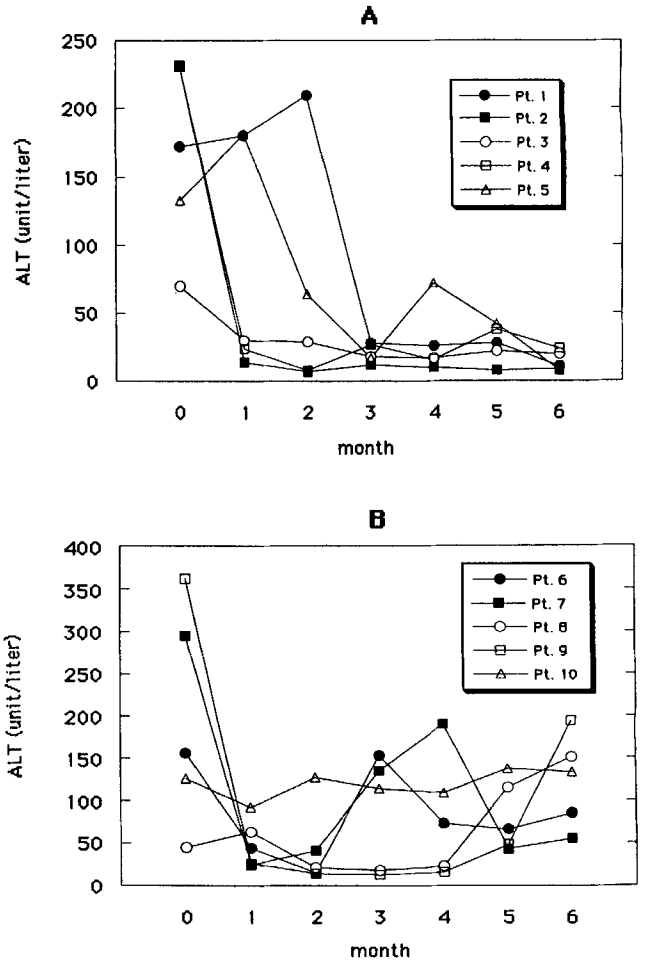

Fig. 1. Serum ALT profiles of the interferon-α-treated HCV carriers. In patients 1 to 5 (complete remission group, **A**), ALT levels dropped to a normal value after 1–4 months of IF-α therapy but in patients 6 to 10 (transient remission group, **B**) the levels did not drop to a normal value after 6 months.

TABLE II. Classification of HCV Types and Effect of Interferon-α Therapy on HCV Markers

Sex/age	Histological diagnosis	EIA	RIBA	Before IF-α treatment HVR PCR	PCR type[a]	HCV amounts (copies/ml)	After 6 months HVR PCR	PCR type[a]	HCV amounts (copies/ml)	Response to IF-α therapy
A. Control group										
1 F/58	CAH[b]	+	+	+	II	10^7	+	II	10^7	
2 M/53	CAH	+	+	+	III	10^6	+	III	10^6	
3 M/69	CAH	+	+	+	II	10^5	+	II	10^6	
4 F/59	CAH	+	+	+	II	10^8	+	II	10^8	
5 F/71	CAH	+	+	+	II	10^8	+	II	10^7	
6 M/52	CAH	+	+	+	II	10^7	+	II	10^8	
7 M/52	CAH	+	+	+	III	10^9	+	III	10^9	
B. Interferon treatment group										
1 F/58	CAH	+	+	+	III	10^5	−		0	complete remission
2 M/27	CAH	+	+	+	II	10^5	−		0	complete remission
3 F/56	CAH	+	+	+	II	10^6	−		0	complete remission
4 M/26	CAH	+	+	+	II	10^5	+	II	10^4	complete remission
5 M/24	CAH	+	+	+	I	10^7	+	I	10^5	complete remission
6 F/62	CAH	+	+	+	II	10^8	+	II	10^8	breakthrough
7 M/55	CAH	+	+	+	II	10^7	+	II	10^8	breakthrough
8 M/53	CAH	+	+	+	II	10^6	+	II	10^7	breakthrough
9 M/67	CAH	+	+	+	II	10^9	+	II	10^8	breakthrough
10 M/44	CAH	+	+	+	II	10^8	+	II	10^8	no response

[a]Genotype of HCV according to Okamoto et al.'s [1993] classification.
[b]Chronic active hepatitis.

5 min at 65°C, 20 units (0.5 μl) of RNase inhibitor and 10 units (1 μl) of AMV reverse transcriptase were added. To reach a final volume of 30 μl, 2 μl of reaction buffer and 6.5 μl of DEPC-treated water were also added. The mixture was incubated for 60 min at 37°C, followed by incubation for 1 min at 99°C to render the enzyme inactive.

PCR amplification was carried out in 50 μl of reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 2.5 mM magnesium chloride, 15 pmole of each primer, 100 mM of each deoxyribonucleotide triphosphates, 0.01% gelatin and 1.5 units of Taq DNA polymerase with an overlay of mineral oil. The reaction was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) as described by Saiki et al. [1988]. Each reaction cycle included denaturation at 94°C for 15 sec, primer annealing at 58°C for 15 sec and primer extension at 72°C for 30 sec. The first PCR was performed for 35 cycles with outer primers and 1μl of the PCR products was used as a template for the second PCR for 30 cycles using inner primers. For genotyping of HCV, a mixture of type-specific primers was used in PCR as in the procedure of Okamoto et al. [1993]. Quantitative-competitive PCR (QC-PCR) was performed using a 5′-untranslated region-deleted mutant as a control template in order to determine the viral titer.

PCR products were subjected to agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized in the presence of ethidium bromide under ultraviolet transillumination.

DNA Sequencing

Amplified cDNAs of HVR in HCV were isolated from agarose gel and purified with glass milk (Gene Clean II kit, Bio 101, La Jolla, CA), and then subcloned by inserting the cDNA into a pT7Blue vector. Plasmid containing cDNA of HVR of HCV was selected by α-complementation.

Three clones from each of the individual patient's plates were selected randomly and plasmid prepared from each clone was used as a template for the DNA sequencing. DNA sequencing was carried out by Sanger and Coulson's [1978] dideoxy method.

Classification of Patients According to IF-α Response

After IF-α therapy for 6 months, patients were classified into three groups: (1) the IF-α responder group in which ALT level became normal, (2) the breakthrough group in which the ALT level was normal during IF-α therapy and then increased again during IF-α therapy, and (3) the nonresponder group in which the ALT level was above normal during IF-α therapy.

RESULTS

Effect of IF-α on Serum ALT Level

ALT levels (IU/L) in 6 of 7 control patients were higher than the normal level (<30 IU/L) for 6 months of the experimental period, but one control patient maintained a normal level after 4 months (data not shown). ALT levels in 5 of 10 patients in the IF-α treatment group fell to normal at 3 months after IF-α treatment, and the levels were maintained at normal after 6 months. However, ALT levels in another five patients in the IF-α treatment group remained at a much higher level than normal after IF-α treatment (Fig. 1). These data indicated that only 50% of the HCV-infected patients responded to IF-α therapy.

Classification of HCV Genotypes in Patients

When the type specific PCR amplification of the core region of HCV was carried out, three different sizes of PCR products were observed. The numbers of patients with types I, II and III were 1 (5.9%), 13 (76.5%), and 3 (17.6%), respectively. Neither type IV nor type V of HCV were detected in the present study (Table II).

Identification of Mutations in HVR of E2/NS1 Domain of HCV

HVR of E2/NS1 domain of HCV was detected in all sera of the untreated control patients by RT-PCR amplification, but HCV RNA was not detected in the sera of 3 IF-α-treated patients (Table II). To confirm the absence of PCR product, QC-PCR for 5′-UTR was undertaken for RNA quantification and did not detect any PCR products. This implied the disappearance of HCV RNA in sera of patients.

The sequence diversity of HVR of HCV in the same serum of a patient (7 in Fig. 1) is shown in Figure 2. Nucleotide sequences of HVR of HCV cDNA in three cloned plasmid DNA samples obtained from individual serum are shown in Figure 3. There were 18 different amino acid sequences in 21 cloned cDNAs of HCV in the control group (3 cloned cDNAs for each patient) at the beginning of the experiment, and after 6 months, 19 different amino acid sequences with 13 new sequences appeared (Figure 3).

Fifteen cloned cDNA of HCV from 5 patients with complete remission (3 cloned cDNAs for each patient) produced 10 different amino acid sequences in the IF-α-treated group at the beginning of the experiment. After IF-α therapy for 6 months, one out of six cloned cDNAs (2 patients) appeared as a new sequence (Fig. 4). At the beginning of the experiment, all 15 cloned cDNA of HCV from the sera of five patients who did not respond to IF-α therapy had different amino acid sequences from each other and six months later, 14 different amino acid sequences appeared of which 12 were new sequences (Fig. 5).

Ten cloned cDNAs obtained from the sera of 3 IF-α-treated patients (6, 7, 10) were selected at random and the nucleotide sequence of HVR was analyzed (Fig. 6).

Six types of amino acid sequences appeared in the HVR of HCV in one patient (6) before the IF-α treatment. At 3 months after IF-α treatment, six different types of amino acid sequences appeared in HVR of HCV, 2 of which were new sequences. HVR of HCV was obtained at 6 months after IF-α treatment; six different types of amino acid sequences in HVR with two new sequences were found.

When a patient (7) carrying HCV with seven different types of amino acid sequences in HVR was treated with IF-α for 1 month, HCV appeared with five different types of amino acid sequences in HVR, of which one was a new sequence. After treatment with IF-α for 3 months, HCV appeared with nine different types of amino acid sequences in HVR, of which 5 were of a new sequence. After treatment with IF-α for 6 months, HCV appeared with three different kinds of amino acid sequences in HVR of HCV without any new sequence.

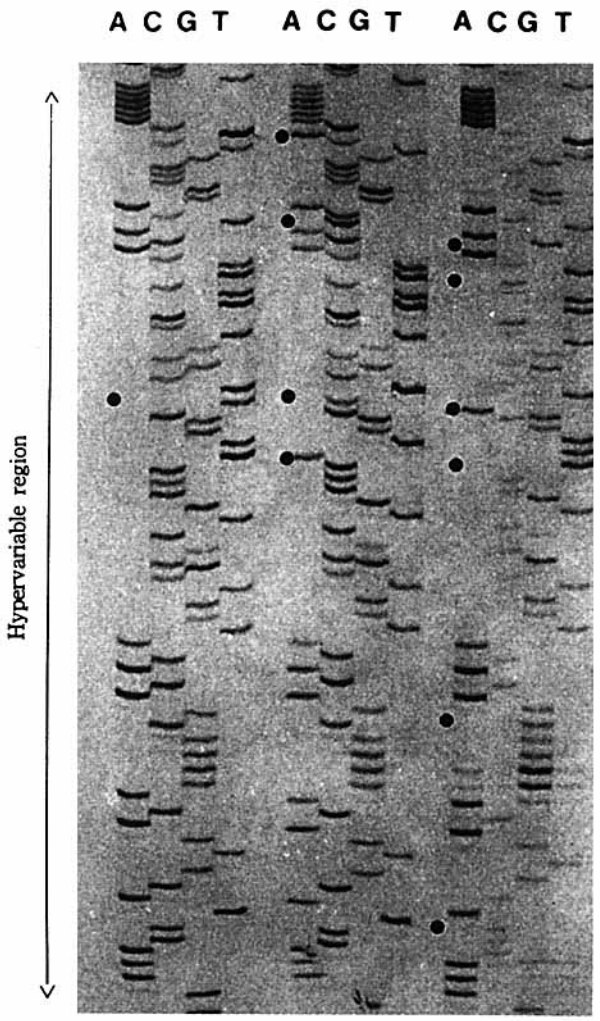

Fig. 2. Nucleotide sequences of cDNA fragments corresponding to HVR of HCV amplified by the reverse transcription-polymerase chain reaction (RT-PCR). The arrow indicates HVR and the dot indicates a different base. DNA sequences in the panel represent the sequences of 3 cloned cDNAs of HCV in the serum of a patient (7 in Fig. 5).

When a patient (10) carrying HCV with seven different types of amino acid sequences in HVR was treated for 6 months with IF-α, HCV appeared with nine different types of amino acid sequences in HVR, of which 6 were new sequences.

DISCUSSION

In the present study, sequence variations of HVR of HCV were analyzed in relation to the therapeutic effect of IF-α on the clearance of HCV in the sera of chronic HCV patients.

Nucleotide sequences in HVR of HCV in sera of chronic HCV patients were variable and the rate of variation was much higher in the IF-α nonresponders as well as in the untreated control patients compared to that in IF-α responders. Direct sequencing of PCR-amplified HVR of HCV DNA fragments was not carried out, and sampling errors may have existed in the present study. But the sequence analysis of HCV cDNA for individual

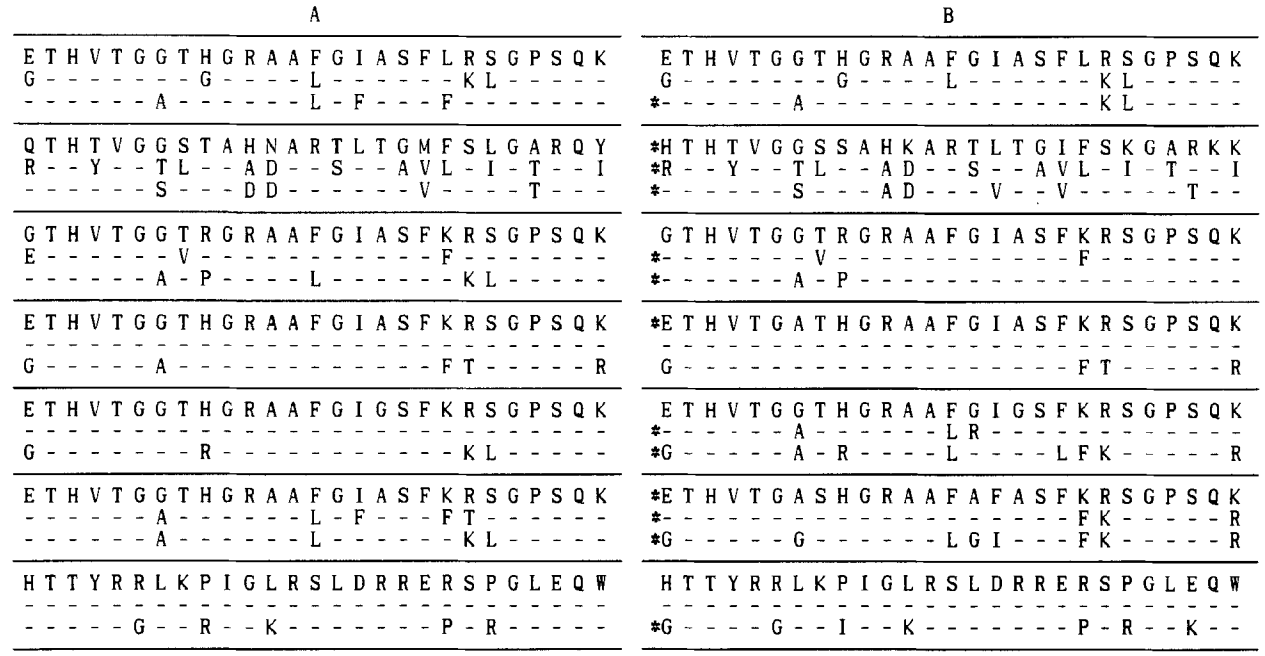

Fig. 3. Amino acid sequences predicted from nucleotide sequences in the hypervariable region of HCV cDNAs amplified by reverse transcription-PCR (RT-PCR) from HCV RNA in sera of patients (control group). Three cloned sequences for each patient are shown. **A** shows amino acid sequences at the beginning of the experiment and **B** shows the sequences after 6 months. Numbers 1 to 7 denote patient's number and the dashed line indicates identical residue to the sequence in the uppermost line. Amino acids in the N-terminus of the E2/NS1 region of HCV are presented. Asterisks denote a newly appeared sequence at 6 months after IF-α treatment.

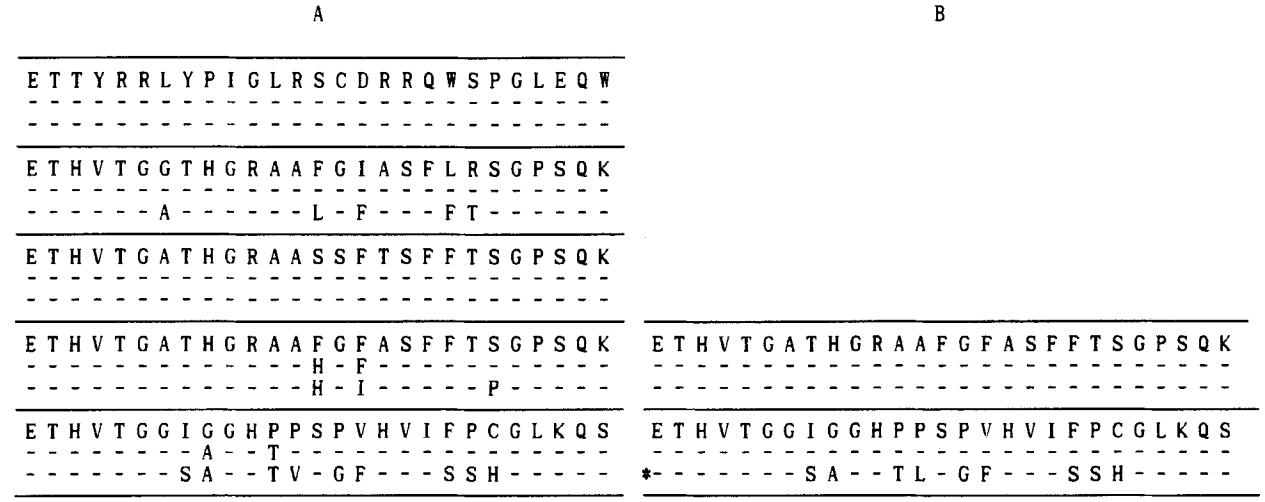

Fig. 4. Amino acid sequences predicted from nucleotide sequences in the hypervariable region of HCV cDNAs amplified from HCV RNA in the sera of HCV-infected patients treated with IF-α for 6 months (complete remission group). Three cloned cDNA for each patient are presented. **A** shows amino acid sequences at the beginning of the experiment and **B** shows the sequences at 6 months after IF-α treatment. Numbers are patient's numbers, and the dashed line indicates the identical residue to that in the uppermost line. Asterisk denotes a newly appeared sequence after IF-α treatment for 6 months.

clones obtained randomly from the whole viral population in the present study might have had less chance of missing the minor variants present.

Recently, Kato et al. [1994] hypothesized that HCV in circulating blood from patients with type C hepatitis might vary in quantity as well as in sequence, perhaps by sequential mutation over time. In contrast to this hypothesis, Weiner et al. [1992] suggested that the HVR1 domain of HCV is subject to immune selection in which antibodies against the well-exposed epitope of the viral envelope protein neutralize a specific virus variant, resulting in the selection of escape mutants favoring a transient escape from the host immune system. According to the immune selection hypothesis, neutralizing

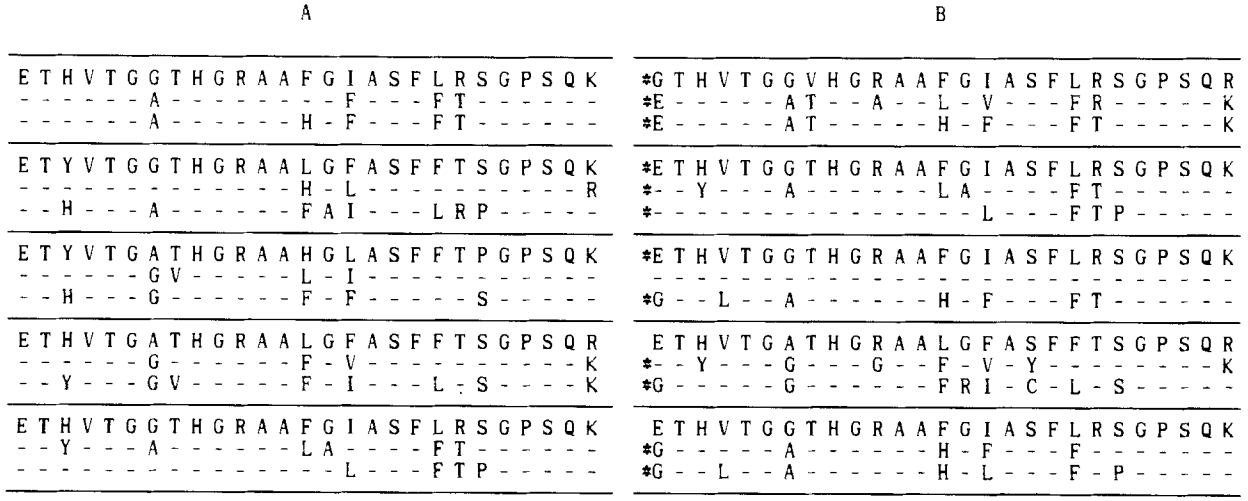

Fig. 5. Amino acid sequences predicted from nucleotide sequences in the hypervariable region of HCV cDNAs amplified from HCV RNA in the sera of HCV-infected patients treated with IF-α for 6 months (transient remission group). Three cloned cDNA for each patient are presented. **A** shows amino acid sequences at the beginning of the experiment and **B** shows the sequences after 6 months. Each number denotes a patient's number and the dashed line indicates identical residue to the sequence in the top. Amino acids in the N-terminus of the E2/NS1 region of HCV are presented. Asterisks denote a newly appeared sequence after IF-α treatment for 6 months.

antibodies against HVR of HCV cause positive selection of escape mutants which may contribute to the predominant presence of new variants. Since patients participating in the present study were diagnosed histologically as chronic active hepatitis, it was assumed that most had been infected with various forms of HCV for a long period of time. Therefore, homologous sequences in HVR of HCV in the present study were not observed (Fig. 3).

HCV-infected patients who received IF-α therapy were divided into two groups: IF-α responders and IF-α nonresponders (Fig. 1). The proportion of the IF-α responders was very similar to that of earlier reports that interferon therapy was effective in about 50% of the patients [Davis, 1990; Weiland et al., 1990; Piccioto et al., 1993].

Normalization of the ALT level in the IF-α responders reflected the reduction of HCV titer in the circulating blood of patients as shown in Table II. Although the humoral antibodies against HCV were not quantified in this study, the reductions in both the HCV titers and in the ALT activities in the IF-α responding patients indicated that IF-α might potentiate the host immune system to counteract HCV replication.

The rate of sequence variation in HVR of HCV in the five IF-α nonresponders was higher than that of the IF-α responder group, suggesting that the host immune system may be involved in determining the sequence variation of the infected HCV. The relatively low HCV titers before the IF-α treatment in the group with complete remission implicates that HCV titer in the serum may be a good parameter for the IF-α response.

These results were consistent with the previous report that the variation of sequence in the amino terminal region of the E2/NS1 of HCV correlates with responsiveness to interferon therapy in viremic patients [Okada et al., 1992]. In their report, amino acid sequences of HCV in the interferon nonresponders varied more than those in the interferon-responding patients during continuous interferon therapy for 1 year. The decrease in number of variants of HCV in patients who responded to IF-α therapy reflected the existence of multiple forms of escape variants of HCV in the IF-α nonresponders.

In the present study, DNA sequencing was carried out for HVR of 10 cloned HCV cDNA obtained from the sera of 3 IF-α nonresponders to determine the possible number of subspecies of HCV with different amino acid sequences in HVR. The data indicated that sequence variations of HVR of HCV prevail in the IF-α nonresponding patients and that the new sequences may appear frequently even after the IF-α treatment. However, these data were not sufficient for the evaluation of the IF-α effect on the generation of immune escape mutant. Kato et al. [1994] reported that the frequent appearance of new variants of HCV happens more often in patients who do not respond to IF-α therapy, and they considered that the continuous appearance of new variants may help the escape of HCV from the host immune system. On the other hand, the sequence evolution of HVR1 in E2/NS1 of HCV caused by specific humoral immune responses has been demonstrated by Doorn et al. [1995] in chimpanzees inoculated with the same genotype 1b strain containing a uniquely predominant HVR1 sequence.

The appearance of a new amino acid sequence in the HVR of HCV in the serum of an IF-α responder (patient 5 in Fig. 4; patients 6 and 7 in Fig. 6) suggests the selection pressure imposed by humoral antibodies, but sampling of a preexisting other variant may have led to this result. It is probable that 10 variants of HCV present in patients and that the many preexisting mutants appeared as new variants in the present experiment. Therefore, further analysis of more cloned HCV cDNA per patient is required.

The present results indicate that HCV maintains its

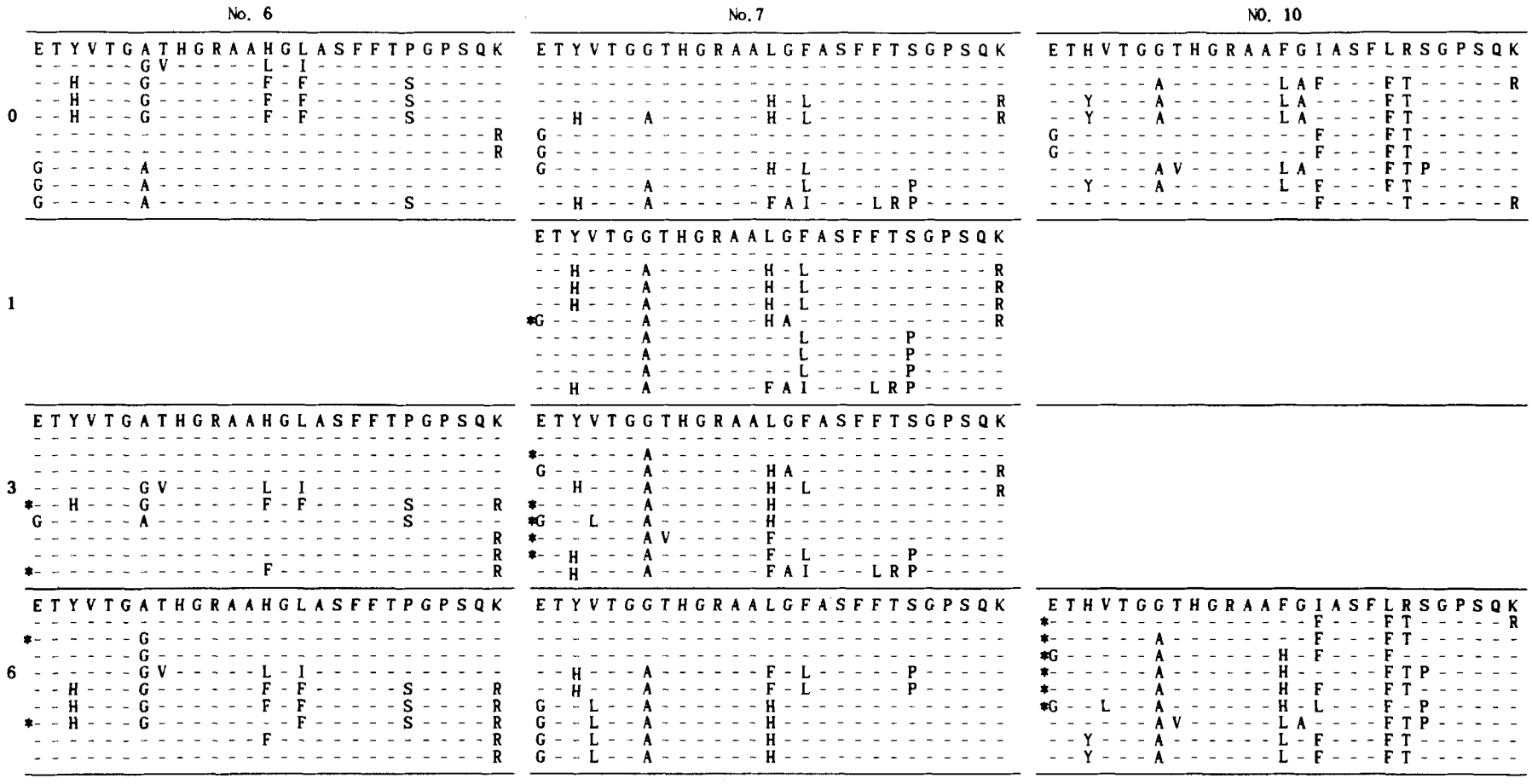

Fig. 6. Amino acid sequences predicted from nucleotide sequences of the hypervariable regions of HCV cDNAs. Ten cloned sequences for each patient are shown. Amino acid sequences of HVR of HCV, which were obtained from three patients (6, 7, 10) treated with IF-α, are presented. The numbers on the left column indicate the period (month) of IF-α treatment. Patient 6 showed no response to IF-α treatment (after 3 and 6 months). Patient 7 responded to IF-α therapy after 1 month, but no response to IF-α after 3 and 6 months was observed. Patient 10 did not respond to IF-α treatment. The amino acid sequences in HVR of HCV were predicted from nucleotide sequences of cDNA of HVR of HCV. IF-α effect was determined by the level of ALT (Fig. 1). A dashed line denotes identical residue to the sequences on the top in each panel. Amino acids are from the N-terminus of E2/NS1 region. Asterisks denote a newly appeared sequence.

chronic state through sequence evolution involving the host immune system, and the appearance of sequence variation in HVR of HCV is related to the therapeutic response to IF-α.

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